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Certified Reference Materials

AOCS 1206-B2

Report of the certification process for

H7-1 Sugar Beet

Second Batch

OECD Unique ID KM-ØØØH71-4

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A2LA ACCREDITED
ISO 17034:2016
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Contents

Abstract 4
Acknowledgements 5
Glossary 6
Introduction 7
Material Processing 7
Trait Verification to Certify Presence of H7-1 7
Certified Value and Measurement Uncertainty 8
Homogeneity 9
Stability 10
References 12

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Abstract

This report describes the preparation and certification of the sugar beet Certified Reference Material AOCS 1206-B2 produced by AOCS Technical Services in 2021. The CRM has been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of sugar beet for transformation events. The sugar beet H7-1 powder was provided by Bayer CropScience LP and KWS SAAT SE & Co. KGaA. It was prepared by grinding the bulk seed at IBRL ay University of Illinois. The certified value of AOCS 1206-B2 was based on the purity of the bulk seed material and with 95% confidence, the true value is ≥ 916 g/kg. The powder was aliquoted and bottled in 27-mL glass headspace vials and sealed under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of H7-1 in AOCS 1206-B2 was verified using event-specific, qualitative PCR analysis by FoodChain ID Testing, LLC, Chantilly, VA (an ISO 17025 accredited laboratory). Homogeneity was verified on random vials of AOCS 1206-B2 using quantitative PCR analysis by FoodChain ID Testing, LLC, Chantilly, VA. CRM samples should be stored in a dry, sealed container at ambient or cooler conditions in the dark.

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Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Crop variety with no history of modern biotechnology and is produced through plant-breeding techniques that rely on selecting and mating parent plants possessing promising traits and repeatedly selecting for superior performance among their offspring
DNA	Deoxyribonucleic Acid
Detection Limit	Lowest level at which target DNA can be detected in a sample and be reliably tested by PCR methods. It is typically expressed as a percentage: the ratio of the number of modern biotechnology derived genomes to the number of crop genomes times 100 percent
EC	European Commission
GMO	Organism that has had genetic sequences modified using molecular-level techniques
ISO	International Organization for Standardization
PCR	Polymerase Chain Reaction: technique used to determine whether a sample of plant tissue contains a particular DNA sequence. PCR relies on primer sets that zero in on a particular target DNA sequence and a special DNA-copying enzyme (DNA polymerase) that makes enough copies of the target sequence for identification and measurement
Qualitative PCR	PCR methods that determine the presence or absence of a specific target DNA sequence at a particular level of detection
Quantitation Limit	Lowest level at which the amount of target DNA sequence in a sample can be reliably quantified. It is typically expressed as the ration of the number of transgenic genomes to the number of crop genomes times 100 percent
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
Trait: H7-1	Resistance to the herbicide glyphosate

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Introduction

Plant genetic modification is an extension of traditional plant breeding. It allows plant breeders to develop crops with specific traits including insect, disease, and herbicide resistance; processing advantages; and nutritional enhancement. An important component for identifying these new traits is a Certified Reference Material (CRM) created from leaf, seed, or grain containing the new trait as well as a CRM created from the conventional counterpart. The European Commission has mandated that from 18 April 2004, a method for detecting a new event derived from transgenic technology and CRM must be available before the EC will consider authorizing acceptance of a new crop derived from transgenic technology. Several nations outside Europe also require grain and ingredients to be labeled above a threshold level before accepting a shipment.

To meet the above regulatory requirements for GMO determination, AOCS 1206-B2 was manufactured from sugar beet according to ISO 17034:2016 and in accordance with EC No 1829/2003, EC No 641/2004 and EC No 619/2011. This CRM is available from AOCS.

Material Processing

The H7-1 sugar beet seed used to prepare AOCS 1206-B2 is hemizygous hybrid seed produced by crossing a non-genetically modified line as female donor and a homozygous H7-1 line as male donor. IBRL at University of Illinois milled ~6 kg of H7-1 sugar beet seed. All of the seed powder was passed through a 710 µm mesh sieve. The seed powder was delivered to AOCS who contracted Illinois Crop Improvement Association for packaging the samples. The powder was aliquoted and bottled in 27-mL glass headspace vials and sealed under a nitrogen gas environment.

Trait Verification to Certify Presence of H7-1

The presence of the H7-1 trait was assessed on 10 random vials of AOCS 1206-B2. AOCS used the Random Number Generator function of Microsoft Excel to select samples for verification of trait presence. Sample numbers that were randomly selected were sent to FoodChain ID Testing, LLC, Chantilly, VA (an ISO 17025-accredited laboratory) for event-specific qualitative PCR analysis to verify the presence of H7-1 in the samples (Table 1).

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Table 1. Trait verification testing on AOCS 1206-B2 H7-1 sugar beet performed by FoodChain ID Testing, LLC, Chantilly, VA (an ISO 17025-accredited laboratory).

AOCS 1206-B2 Sample	Trait H7-1 Presence
Sample # 25	Positive
Sample # 91	Positive
Sample # 113	Positive
Sample # 176	Positive
Sample # 230	Positive
Sample # 257	Positive
Sample # 314	Positive
Sample # 349	Positive
Sample # 394	Positive
Sample # 445	Positive

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 1206-B2 was assessed by FoodChain ID Testing, LLC, Chantilly, VA (an ISO 17025-accredited laboratory). A total of 96 sugar beet seeds were subjected to individual seed testing for the presence of H7-1 by event-specific qualitative PCR. 96 of the 96 seeds tested positive for the presence of H7-1.

Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponded to the lower bound of true % purity. The % purity in the sample was 100%, when 96 seeds were tested. Using a 95% confidence level, the true % purity of the H7-1 seed lot was at least 91.6%. Consequently, with 95% confidence, the true value is ≥ 916 g/kg.

The measurement uncertainty (U_{CRM}) is the expanded uncertainty with a coverage factor of 2 and a confidence level of 95%. It is obtained by combining the uncertainties from the purity assessment ($u_{char,rel}$), the homogeneity assessment ($u_{bb,rel}$), the transport stability assessment ($u_{sts,rel}$) and the long-term stability assessment ($u_{lts,rel}$):

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$$u_{CRM,rel} = \sqrt{u_{char,rel}{}^2 + u_{bb,rel}{}^2 + u_{sts,rel}{}^2 + u_{lts,rel}{}^2}$$

$$U_{CRM} = 2 \times u_{CRM,rel} \times 1000\ g/kg$$

Consequently, the expanded measurement uncertainty for AOCS 1206-B2 is -84 g/kg.

Homogeneity

The homogeneity of AOCS 1206-B2 is related to the purity of the seeds. 96 out of 96 seeds tested positive for the H7-1 sugar beet event by event-specific qualitative PCR. Based on the sample purity of 100%, as determined using SeedCalc8, the batch was expected to be homogenous.

To further confirm homogeneity, ten vials of AOCS 1206-B2 (randomly selected as described above) were provided by AOCS to FoodChain ID Testing, LLC, Chantilly, VA (an ISO 17025 accredited laboratory). Homogeneity was assessed using the H7-1 event-specific quantitative PCR method (Mazzara et al., 2013). For each of the 10 CRM vials analyzed, there were 2 independent DNA extractions. Each DNA extraction was subject to 3 quantitative PCR replicates. The data produced from these quantitative PCR reactions provided the numeric copies of H7-1 and the numeric copies of glutamine synthetase (GS), a sugar beet endogenous reference gene. The property value assessed here is defined as the ratio between copies of the H7-1 target and copies of the glutamine synthetase (GS) target.

The quantitative PCR data was used to evaluate the within-unit and between-unit homogeneity of AOCS 1206-B2 to ensure that the property value is valid within vials of CRM and between vials of CRM. The CRM will be determined to be homogeneous if the with-in relative standard deviation (RSD_w) and between-unit relative standard deviation (RSD_b) are both ≤ 20%.

Quantification of between-unit (vial/sample) inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation from the within-unit variation. Preliminary analysis showed that there is no significant variation between the two DNA extractions within each vial, so the DNA extraction effect was not considered in

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the analysis. That is, all replicates for each vial were treated as independent observations regardless of which DNA extraction they were from.

Within-unit relative standard deviation ($\mathrm{RSD_w}$), between-unit relative standard deviation ($\mathrm{RSD_b}$) were calculated as:

Within-unit RSD: $RSD_w = \frac{\sqrt{MS_{within}}}{\bar{y}}$

Between-unit RSD: $RSD_b = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$

where,

$\mathrm{MS_{within}}$ within-unit mean square from an ANOVA
$\mathrm{MS_{between}}$ between-unit mean square from an ANOVA
$\bar{y}$ mean of all results of the homogeneity study
n mean number of replicates per unit (5.9 for H7-1)

Table 2. The within-unit relative standard deviation (RSD_w), and the between-unit relative standard deviation (RSD_b) for vials of AOCS 1206-B2.

CRM	RSD_w [%]	RSD_b [%]	$u^*_{bu,rel}$ [%]
AOCS 1206-B2	8.4	n.c.[1]	4.0

[1]n.c: RSD_b cannot be calculated as $\mathrm{MS_{between}} < \mathrm{MS_{within}}$. In this situation, maximum hidden inhomogeneity ($u^*_{bu,rel}$) is provided as an alternative

This confirms the homogeneity of AOCS 1206-B2.

Stability

Time, temperature and light are regarded as the most relevant influences on the stability of CRM (Linsinger, et al., 2001). The influence of light is mitigated by shipping and storing the vials in boxes, thus minimizing the possibility of degradation due to light. The influence of temperature is mitigated by storing the vials in a temperature-controlled room, and shipping vials at ambient temperature.

The effect of temperature and time are investigated.

A transport (short-term) stability study is conducted to assess the stability of maize CRM during transport. The temperature and time conditions in the study cover the typical

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conditions and the not so rare situations. The outcome of the study is considered transferable to other CRMs of similar property. Samples were subject to 3 different temperatures (4 ℃ (fridge), 25 ℃ (ambient), 60 ℃ (oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 ℃ (fridge) and 25 ℃ (ambient) for 4 weeks. The estimated uncertainty contribution from transport (short-term) stability is 1.0%.

A long-term stability study is conducted to assess the stability of maize CRM during storage. Samples are stored at 25 ℃ (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperate studied is 25 ℃ and the length of time to be studied is 10 years. The outcome of the study is considered transferable to other CRMs of similar property. In the initial 1-year stability study, samples were subject the storage condition for 4 different durations (0, 1, 3, 6 and 12 months). The study concluded that samples are stable at 25 ℃ (ambient) for 12 months. The estimated uncertainty contribution from long-term stability is 0.42%.

CRM stability over time will be analyzed by repeating the homogeneity study described above at a chosen shelf life of approximately every 24 months. The 24-month shelf life of CRM is chosen because the influence of analytical variation can be reduced by increasing the length of the stability study (Linsinger, et al., 2001).

The initial ratio between the number of copies of the GM event and the number of copies of the endogenous reference gene from the homogeneity study will establish the base line for the stability study. The ratio at each 24-month interval will be compared to the ratio established in the homogeneity study. The CRM will be determined to be stable if the variability of the ratios, determined as relative standard deviation (RSD) is ≤ 20%.

Stability of these CRMs has been listed as 2 years from the certification date. The materials were processed and are stored at ambient temperature, under nitrogen gas, in 27-mL glass headspace vials. These materials are expected to be stable for longer than the estimated expiration date. The stability of the powder material will be reevaluated at time of expiration. If the samples are determined to be stable, the certificates will be extended.

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References

FoodChain ID Testing, LLC, 4150 Lafayette Center Drive, STE. 600, Chantilly, VA 20151; Telephone: 1-888-229-2011 or 1-641-472-9979 ext. 0; testing@foodchainid.com

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